Identification of a differentially-expressed message associated with colon cancer liver metastasis using an improved method of differential display

Timothy J. Yeatman* and Weiguang Mao

Department of Surgery, H. Lee Moffitt Cancer Center, University of South Florida, 12902 Magnolia Drive, Tampa, FL 33612, USA

Received July 24, 1995; Accepted August 20, 1995

Colon cancer results in nearly 50 000 deaths per year in the United States alone (1). The majority of these patients succumb to liver metastases derived from the primary tumor. We now know that this metastatic process is not random, but, rather, is based on multiple specific factors such as adhesion molecules, growth factors and invasive enzymes which must be produced by the metastatic cell (2). Methodology striving to identify differentially-expressed messages is critical to the process of identifying the genes responsible for the metastatic phenotype.

We initially employed the method of differential display (3-5) to identify differentially expressed messages in three human colon cancer cell lines. Differential display (DD) is a recently-described methodology for identifying differentially-expressed genes that has multiple advantages over classical methods such as subtractive hybridization. DD, however, is often unsuccessful (6,7) in producing an adequate probe for Northern blot, required for confirmation of any differences in gene expression that may exist. Often, a great deal of time is spent screening many different probes for evidence of differential expression. We postulated that many of the failed Northern analyses may be secondary to the inherent problems of using very small probes for either rare or large messages—both of which are difficult to detect.

To study the problem of colon cancer liver metastasis, we selected for study one human colon cancer cell line (KM12 C) known to be poorly metastatic to the liver in a nude mouse model of intrasplenic injection, as well as two related highly-liver metastatic sublines (KM12 SM and KM12 L4A) that were derived from KM12C (generous gift from I. Fidler) (7). Although we were able to identify apparent differences in the DDs performed, we were not able to decisively confirm these differences in Northern blots (Fig. 1). Northern blots produced smears rather than discrete bands and, consequently, were difficult to interpret.

To circumvent these problems, we devised a strategy whereby polymerase chain reaction (PCR) products could be used directly in a ribonuclease protection assay (RPA) as well as for automated cycle sequencing, thereby eliminating the initial cloning requirements associated with standard approaches, yet taking full advantage of the exquisite sensitivity of the RPA. With this revised methodology, we were able to successfully identify a message that was overexpressed in colon cancer cells that are

Figure 1. Northern analysis of the CO-10 probe generated from differential display. CO-10 was labeled by the random prime technique to high specific activity yet produced only smears on Northern analysis when hybridized to 20 µg/lane of KM12 C (C), KM12 SM (SM) and KM12 L4A (L4A) total RNA.

highly-metastatic to the liver when compared with cells that are non-metastatic.

Specifically, the DD was performed with an anchored T₁₁ oligonucleotide primer (5'-AAGC-T₁₁C-3') and an arbitrary 13mer oligonucleotide primer (5'-AAGCTTAGAGGCA-3') (Genhunter) as previously described (3). Copy DNA sequences (cDNA) appearing to be differentially produced from total RNA, were reamplified by PCR with the same primer sets. A second round of amplification was then performed following gel purification of the PCR products, using a modified 3' oligonucleotide primer that incorporated a T7 RNA polymerase binding site on the 5' end (5'-GAATTCTAATACGACTCACTA-TAGGGAAGCT₁₁C-3'), as well as restriction sites for cloning such that all PCR reamplification products would have one cDNA strand (minus strand) with a T7 binding site. PCR reamplification conditions with the modified primer were no different from the unmodified primer (94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles followed by 72°C for 5 min). This permitted direct use of these PCR products (~50-100 ng) as template for the T7 RNA polymerase resulting in production of a riboprobe (CO-10) labeled to high specific activity with [³²P]CTP.

An RPA (8-10) was then performed as using a commercial kit (Ambion) using CO-10 with confirmation of a differentially expressed message using β-actin (125 bp) as an internal control

* To whom correspondence should be addressed

Figure 2. Ribonuclease protection assay using CO-10 riboprobe generated from differential display. The CO-10 probe, when added to each RNA sample, generated an ~300 bp protected fragment (after RNase treatment) that represented over-expressed message levels in the highly metastatic KM12 L4A (L4A) colon cancer cell line versus the poorly metastatic KM12 C (C) cell line. Two concentrations of sample RNA were tested (10 and 5 μ g). Internal β -actin (125 bp) controls demonstrate relatively equal loading of total RNA in each lane. Additional controls shown are yeast RNA (10 μ g) with and without RNase.

(Fig. 2). Labeled probe (~80,000 c.p.m.) was hybridized in solution with 10 μ g of total RNA from each cell line as well as with 10 μ g of yeast control RNA with and without RNase. We believe that the clarity of these results is secondary to the nearly 10-fold increase in sensitivity afforded by use of an RPA. Direct cycle sequencing was then performed on the same PCR product using a 5'-fluorescein modified T7 primer which permitted sequencing of the entire cDNA product. Sequencing confirmed strong homology of CO-10 with a known human gene (MB-1), known to code for a surface immunoglobulin molecule (11). While further study is needed, our differentially-expressed message may potentially represent a novel adhesion molecule of the immunoglobulin supergene family.

In conclusion, analysis of total RNA from three related cell lines by DD resulted in a cDNA that appeared to be produced from highly metastatic colon cancer cells but not from poorly metastatic cells. Initial attempts at Northern analysis with a

CO-10 riboprobe produced a blot with poor visualization of hybridization. However, further analysis by RPA demonstrated a 3.1-fold increase in levels of mRNA expression of the CO-10 probe which is ~300 bp in size.

By simple modification of the DD primer sets, we were able to rapidly confirm presumed differences in gene expression, as well as directly sequence the same PCR products. When riboprobes of different sizes are used, more than one probe may be tested at the same time, along with internal controls, permitting economic use of valuable RNA samples as well as direct comparison of multiple probes in the same experiment. These modifications represent a significant improvement in the standard approaches to analysis of DD products that require an initial cloning step, and are often fraught with false positive results due to low copy messages or poorly-hybridizing cDNA probes.

ACKNOWLEDGEMENTS

This work was supported by grants ACS 6120069L0 and CA65512-01. We thank Deepak Agrawal, PhD, for his thoughtful discussions in the preparation of this manuscript.

REFERENCES

- 1 Boring C.C. (1994) *CA Cancer Clin. J.* **44**, 7-26.
- 2 Yeatman T.J., Nicolson G.L. (1993) *Semin. Surg. Onc.* **9**, 256-263.
- 3 Liang P., Pardee A.B. (1992) *Science* **257**, 967-971.
- 4 Liang P., Averboukh L., Keyomarsi K., Sager R., Pardee A.B. (1992) *Cancer Res.* **52**, 6966-6968.
- 5 Bauer D., Muller H., Reich J., Riedel H., Ahrenkiel V., Warthoe P., and Strauss M. (1993) *Nucleic Acids Res.* **21**, 4272-4280.
- 6 Liang P., Averboukh L., Pardee A.B. (1993) *Nucleic Acids Res.* **21**, 3269-3275.
- 7 Bao-Ling A., Wright G.L. Jr. (1995) *Proc. Annu. Meet. Am. Assoc. Cancer Res.* **36**, A150.
- 8 Myers R.M., Larin Z., Maniatis T. (1985) *Science* **230**, 1242-1246.
- 9 Calzone F.J., Britten R.S., Davidson E.H. (1987) *Methods Enzymol.* **152**, 611-632.
- 10 Lee J.J. and Costlow N.A. (1987) *Methods Enzymol.* **152**, 633-648.
- 11 Ha H., Barnoski B.L., Sun L., Emanuel B.S., Burrows P.D. (1994) *J. Immunol.* **152**, 5749-5757.